The branching of life: human iPSC-based angiogenesis-on-chip

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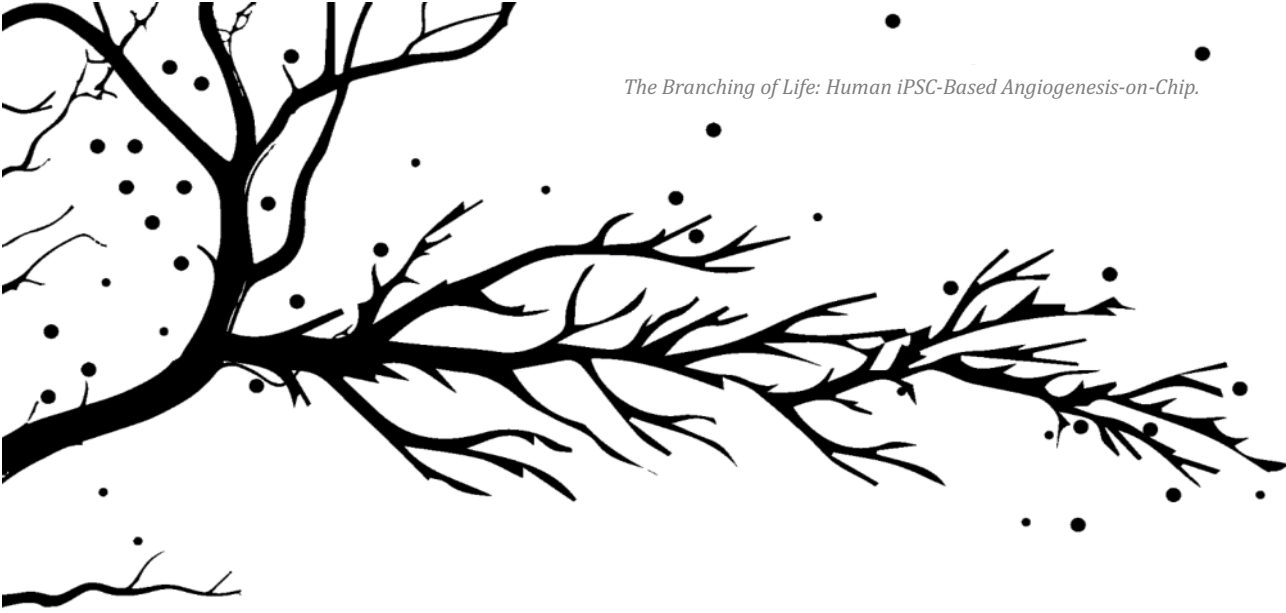

Chapter 2

Angiogenesis-on-chip Based on hiPSC-derived Endothelial Cells for High-Content Screening

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ABSTRACT

Human induced pluripotent stem cell-derived endothelial cells (hiPSC-ECs) are increasingly used to model angiogenesis. In some cases, the models would benefit from coupling to microfluidic devices where angiogenic sprouts could develop in 3D and could be subject to fluid flow. To realise 3D angiogenesis-on-chip (AoC) assays able to recapitulate vessel sprouting, we developed a protocol using hiPSC-ECs in combination with either type I collagen or fibrin matrix in a commercially available platform. Use of the protocol to evaluate the angiogenic phenotype was demonstrated for different hiPSC-EC lines. Robust hiPSC-EC sprouting was observed in both matrices, although there were significant differences in quantified biological features in each case. Nevertheless, the AoC model is ready for use to assess angiogenesis by hiPSC-ECs for multiple applications.

Basic Protocol 1: Angiogenesis-on-chip set-up

- **Part 1:** Preparation and gel filling of Fibrin matrix
- **Part 2:** Preparation and gel filling of type I collagen matrix (Optional)
- **Part 3:** Fibronectin coating of the upper media channel
- **Part 4:** hiPSC-ECs seeding and monolayer formation

Support Protocol 1: Preparation of hiPSC-Derived Endothelial Cells for AoC culture

Basic Protocol 2: 3D sprouting angiogenesis

- **Part 5:** Angiogenic stimuli
- **Part 6:** Characterization and quantification of the hiPSC-EC sprouts
  - **Part 6.1:** Fixation and immunofluorescence staining
  - **Part 6.2:** high-content imaging
- **Part 7:** Analysis

Support Protocol 2: Quality control of the hiPSC-ECs-based platform

Keywords: Sprouting angiogenesis, human induced pluripotent stem cells, vessel-on-chip, endothelial cells.

INTRODUCTION

Angiogenesis is a tightly regulated process in which new vessels sprout from pre-existing vasculature, resulting in new vessel growth (Potente and Mäkinen, 2017). Physiological angiogenesis is orchestrated by coordinated perfusion and gradient-driven activation of a series of receptors in quiescent endothelial cells (ECs). 3D angiogenic sprouting EC models in microfluidic devices, referred to as angiogenesis-on-chip (AoC), have been engineered to promote capillary growth and the formation of lumen-like elongated tubal structures. AoCs have been engineered to allow sensing of angiokine gradients, EC signaling, tip/stalk cell specification, EC proliferation, migration, and invasion in a single assay (van Duinen et al., 2019a; van Duinen et al., 2019b; Kim et al, 2016; 2021). Despite showing increased physiological relevance over conventional 2D models such as the spatial organization towards a gradient source (Lee et al., 2024), current AoC models carry several limitations: they may incorporate cells derived from primary tissues such as human umbilical vein ECs (HUVECs) which may vary from batch-to-batch and donor-to-donor, and are not repeatedly available from the same source. The availability of human induced pluripotent stem cells (hiPSC), derived by reprogramming from somatic cells, can be differentiated to any cell types of the body, including endothelial cells (ECs). hiPSC-ECs can thus be derived repeatedly from the same hiPSC source or from different donors, opening new frontiers for modeling disease (Williams and Wu, 2019; Duinen et al., 2020; Natividad-Diaz et al., 2019; Bezenah et al., 2018). hiPSC-ECs have been used to form macro- and microvasculature (Orlova et al., 2018; 2022; Bulut et al., 2022; Williams and Wu, 2019) and have the potential to emulate angiogenic responses and test anti- or pro-angiogenic compounds.

Here, we describe a two-part protocol for (1) setting up AoC, incorporating hiPSC-ECs on a commercially available microfluidic device (Basic Protocol 1) and stimulating the hiPSC-ECs to generate sprouts in 3D (Basic Protocol 2) within a type I collagen or fibrin extracellular matrix (ECM). The preparation of hiPSC-ECs prior to the incorporation into AoC model is described in Support Protocol 1. This provides guidelines to create the complex environment for 3D sprouting angiogenesis while preserving technical simplicity. In addition, methods for characterizing the vessel sprouts by immunofluorescence using automated confocal imaging and subsequent downstream

analysis are provided. We have included quality control metrics for the platform which are critical for obtaining optimal readouts (Support Protocol 2). Together, the methods described contribute to a) overcoming some of the current bottlenecks, and b) providing insights to study angiogenesis in microphysiological systems using hiPSC-ECs.

BASIC PROTOCOL 1: Angiogenesis-on-Chip SET-UP

This protocol describes a vessel-on-chip method based on hiPSC-ECs to study angiogenesis quantitatively employing gravity-driven microfluidic devices. We compared two independent ECMs in the chip: Fibrin (Part 1) and type I collagen (Part 2), both of which can be used for sprouting angiogenesis using hiPSC-ECs. The first step is the preparation of hiPSC-ECs as described in Support Protocol 1 (Fig. 1A). The second step is setting up the chip (Fig. 1Bi), which includes ECM preparation and filling (Part 1 or 2), surface coating of upper media channel (Part 3, Fig. 1Bii, II) and finally, seeding hiPSC-EC to create the primary vessel in the upper media channel (Part 4, (Fig. 1Biii, III-VI). We used the idenTx 40® plate (AimBiotech, Singapore), which consists of a central gel channel flanked by two media channels that allow 40 replicates per experiment. The handling of the plate was performed according to the manufacturer's recommendations (General idenTx40 handling Protocol v1.3, Aimbiotech) and the angiogenesis protocol (Angiogenesis Protocol v3.1, AimBiotech) with multiple modifications.

Part 1: Preparation and gel filling of the Fibrin matrix.

Materials

- Fibrinogen bovine plasma (Sigma-Aldrich, cat. F8630-5G)
- Thrombin (Sigma-Aldrich, cat. T4648-10KU)
- Endothelial Cell Growth Medium 2 (EMG-2) (PromoCell, cat. C-22211, see recipe section)
- Phosphate-buffered saline Dulbecco's formula (DPBS, 1X, Life Technologies, Cat. 70011044)

- IdenTx 40 plate (Aim Biotech Pte Ltd, cat. IDTX40)
- Puradisc 30 0.22-µm membrane filter (Cytiva, cat. 10462200)
- Ice bucket with ice or cooling rack for Eppi® tubes

- 1.5 ml Eppendorf® tubes
- P20 pipette.

Procedure

1. Prepare 5 mg/ml fibrinogen solution (see recipe section).

Perform all procedures in cold using ice or a cooling rack.
Waiting time: 3 hours.

2. Add 40 µL thrombin (50 U/ml stock concentration) to 960 µl EMG-2 to reach a concentration of 2 U/ml thrombin
3. Add 25 µl of the EMG-2-Thrombin mix to one of the 25 µl fibrinogen aliquots (final concentration of fibrin 2.5 mg/ml)

Mix gently, no more than 5 times to avoid excessive polymerization.

4. Place the idenTx40 plate on the cooling block for idenTx plates (optional)
5. Draw the fibrin mix with a P20 pipette and inject 5 µl of the solution manually into the inlet of the gel channel using a P20 pipette (Fig. 1B, i).

Use one aliquot of fibrinogen mix for 3-5 channels and discard the leftover.

6. Repeat steps 3-5 to complete the plate.
7. Add 7 ml of dH_2O or DPBS to the water reservoir of the plate.
8. Remove the plate from the cooling block and place it at room temperature for 15 min to allow fibrin polymerization.

Part 2: Preparation and gel filling of type I collagen matrix (Alternative)

Materials

- Type I collagen master mix (1.5 mg/ml (w/v), see recipe section)
- type I collagen, rat tail (5mg/ml, R&D Systems, cat. 3447-020-01)
- Medium 199 (10X, Gibco, Life Technologies, cat. 21180-021)
- $NaHCO_3$ (7.5 %, Gibco, cat. 25080-60)

- NaOH solution (1 M)
- HEPES (1M) (Gibco, Cat. 15630-080)
- Sterile deionized water (Gibco, cat.15230-089)
- DPBS (1X, Life Technologies, Cat. 70011044)

- IdenTx40 plate (Aim Biotech Pte Ltd, cat. IDTX40)
- Ice bucket with ice or cooling rack for Eppi® tubes
- Cooling Block for idenTx and organiX Plates (Aim Biotech, cat. CBLK)
- 2 ml Eppendorf® tubes (Eppendorf, cat. 0030123344)
- Eppendorf® Multipette E3 pipette (Eppendorf, cat. 4987000010)
- Combitips Advanced® 0.2 ml (Eppendorf, cat. 0030089626)

Procedure

9. Prepare the 1.5 mg/ml type I collagen master mix (see recipe section)
10. Keep the master mix on ice.
11. Place the idenTx40 plate on the cooling block for idenTx plates.
12. Draw 200 µl of the type I collagen master mix with a 0.2 ml Combitip®

Set an aspiration speed of 5 using the Eppendorf® Multipette E3 pipette.

13. Inject 8 µL into the inlet of gel channels using the 0.2 ml Combitip® (Fig. 1B, i).

Set a dispensing speed of 5 using the Eppendorf® Multipette E3 pipette.
It is possible to fill in 20 gel channels using one full Combitip®.

14. Repeat steps 11 and 12 to complete the plate.
15. Add 7 ml of dH_2O or DPBS to the water reservoir of the plate.
16. Place the plate at 37 °C for 15 min.
17. Inspect channels under the microscope to confirm the polymerization of the type I collagen fibers.

Part 3: Fibronectin coating of the upper media channels.

Materials

- Fibrin- or type I collagen-filled idenTx40 chips.
- 50 µg/ml Fibronectin solution (see recipe section)
- Fibronectin (1 mg, Sigma-Aldrich, Cat. no. F1141-1MG, Lot SLBX2646)
- DPBS (1X, Life Technologies, Cat. no. 70011044)
- Endothelial Cell Complete Growth Medium (EC-CGM; see recipe section)

- 2 ml Eppendorf® tubes (Eppendorf, cat. 0030123344)
- Eppendorf® Multipette E3 (Eppendorf, cat. 4987000010)
- Combitips Advanced® 0.2 ml and 5 ml (Eppendorf, cat. 0030089626)

Procedure

18. Prepare the 50 µg/ml fibronectin solution (see recipe section).
19. Inject 10 µl fibronectin coating solution (50 µg/ml) directly in the top right media channel inlet using the Eppendorf® Combitips Advanced® 0.2 ml (Fig. 1B, ii, II).
20. Repeat step 1 in all top media channel inlets.
21. Inject 10 µl of DPBS into all bottom media channel inlets.

Inspect the media channels under the microscope to check their permeability. During the addition of the fibronectin solution, the liquid flows through the medium channel, and a droplet in the opposite outlet (upper left) can be observed.

22. Place the plate for 1 hour at 37°C.
23. Flush the coating solution by adding 15 µl of EC-CGM directly in the top right media inlets.
24. Add 50 µl of EC-CGM to all top and bottom left media ports with a 5 ml Combitip Advanced®.
25. Place the plate for 1 hour at 37°C.

Waiting step of 1 hour. Keep at 37°C under sterile conditions. During the pause, carry out Support Protocol 1 (steps 10-19) to prepare the hiPSC-EC suspension, or store the plate to be used the next day.

Part 4: hiPSC-ECs seeding and monolayer formation

Materials

- Type I collagen-filled or fibrin-filled, fibronectin-coated idenTx40 plate.
- EC-CGM (see recipe section)
- 5x10^6/ml hiPSC-EC suspension.

- Eppendorf® Multipette E3 pipette (Eppendorf, cat. 4987000010)
- Combitips Advanced® 0.2 ml (Eppendorf, cat. 0030089626)
- Combitips Advanced® 5,0 ml (Eppendorf, cat. 0030089669)
- OrganoFlow® Rocker (Mimetas, cat. MI-OFPR-L)

Procedure

26. Aspirate all of the medium from the reservoirs.

Do not aspirate the medium inside the media channel inlets but only from the reservoirs.

27. Inject 10 µL of cell hiPSC-EC suspension directly into the inlets of the upper left media channel using Eppendorf® Combitips Advanced® 0.2 ml (dispensing speed: 4) (Fig. 1B, iii).
28. Leave the plate on a flat surface for 5-10 min without moving at room temperature.
29. Check cell density under a light microscope using a 10X objective.

Cell density must be similar to that in Fig.1B, III-IV and Fig.1B, V-VI, immediately after seeding and 24 hours post-seeding, respectively. Adjust cell density if needed by adding more of the cell suspension. See troubleshooting guidelines (table 6).

30. Place it at 37°C and 5% CO2 for 2 hours to allow cell attachment.

Waiting step of 1-2 hours. The cell seeding step can be also performed manually with a P20 pipette as well as the cell density adjustment.

31. Check cell attachment under the microscope.

32. Add 50 µL of EC-CGM on each side of the upper media channel ports.
33. Place the plate on the rocker to subject the hiPSC-ECs to transversal flow (5° angle x 8 min interval) for 24 hours (Fig. 1B, iv).

Step 33 is optional. However, it is highly recommended as transversal flow using the rocker enhances monolayer formation and confluency.

SUPPORT PROTOCOL 1: Preparation of hiPSC-Derived Endothelial Cells for AoC culture

This two-step section first describes the hiPSC-EC culture before setting up the AoC assay. Details of the hiPSC lines and batches used are given in Table 1. Control hiPSC-ECs were differentiated and characterized as described previously (Orlova et al., 2018; 2014) The starting density is cell line dependent (Table 1), and the usage criteria were established at ~80% confluency (Fig. 1A, I). High cell density is key for the functionality of the assay and its reproducibility: 5x10^6 hiPSC-ECs/ml is needed to obtain a confluent monolayer after 24 hours in the microfluidic device.

Table 1. Details of the hiPSC-ECs used for protocol optimization.

	hiPSC-EC	#Batches	#RRID	Starting format
CTRL#1	NCRM1	2	CVCL_1E71	15 cm^2 in T75 or 6x6-well plate (3 days)
CTRL#2	LUMC0099iCTRL	2	CVCL_UK77	25 cm^2 in T75 (3 days) or 15 cm^2 4x6-well plate (3 days)
CTRL#3	LUMC0020iCTRL6.4	2	CVCL_ZA25	15 cm^2 in T75 or 6x6-well plate (3 days)

Materials

- Gelatin-coated T75 or 6-well plate (see recipe section)
- Cryopreserved hiPSC-ECs on day 10(Orlova et al., 2018; 2014)
- Endothelial Cell Serum-Free medium (EC-SFM; Gibco, cat. no. 11111-044)
- EC-CGM (see recipe section)
- DPBS (1X; Life Technologies, cat. no. 14-190-144)
- TrypLE™ Express Enzyme, no phenol red (1X, Gibco, ThermoFisher Scientific, cat. A12177-01)

- 6-well culture plate Costar® (Corning, cat. no. 3506) or T75 flask CELLSTART® (Greiner, cat. no.658170)
- 15-ml tubes (Corning Falcon, cat. no. 352097)
- 1.5-mL microcentrifuge tubes (Eppendorf, cat. no. 0030 120.086)
- Water bath at 37 °C
- Tissue culture incubator at 37 °C, 5% CO_2

Procedure

1. Coat with gelatin the 6-well plate or T75 for 1 h at room temperature before cell seeding.
2. Prewarm the EC-SFM and EC-CGM media.
3. Thaw hiPSC-ECs in the water bath at 37 °C for <1 min.
4. Add the thawed cell suspension immediately to 6 ml of EC-SFM in a 15-ml tube.
5. Centrifuge the tube for 3 min at 300 x g at room temperature.
6. Aspirate the supernatant carefully.
7. Resuspend the cell pellet in 12 ml of EC-CGM.
8. Add the 12 ml of the cell suspension to a previously gelatin-coated T75 flask or 2 ml of the cell suspension to each well of a 6-well plate.
9. Place the plate/flask at 37°C and 5% CO2 for 3-5 days (Table 1).
10. Prewarm the EC-SFM and EC-CGM media.
11. Aspirate the media carefully.
12. Wash the monolayer of hiPSC-ECs once with 5ml 1X DPBS.
13. Trypsinize the cells by adding 4 ml of TrypLE for three minutes at RT.
14. Inspect the cell detachment by observing the turbidity of the media.
15. Add a two-fold volume of EC-SFM.
16. Centrifuge at 300 g x 3 min.
17. Resuspend the cell pellet in EC-CGM to obtain 5x10^6 hiPSC-ECs/ml.
18. Pipette up and down (5-10 times) with a 1 ml tip.
19. Transfer the cell suspension to a 1.5 ml sterile Eppi® tube.

BASIC PROTOCOL 2: 3D SPROUTING ANGIOGENESIS

This protocol is divided into three steps. The first step is the induction of sprouting angiogenesis from the intact hiPSC-EC monolayer in response to "angiokine" stimuli. Angiokines are molecules with angiogenic activity (Part 5). Here, we used VEGF, S1P, and bFGF, to activate the VEGFR2, S1PR,and FGFR signaling respectively, (Apte et al., 2019; Cartier et al., 2020; Lupino et al., 2019; Chen et al., 2021; Li et al., 2022), and the y-secretase inhibitor DAPT to block the activation of NOTCH pathway (Dong et al., 2021; Eelen et al., 2020; Potente and Mäkinen, 2017) to induce angiogenic responses on hiPSC-ECs (Fig. 1C, VII-XII). The combination of these angiokines can maintain the microphysiological system at the lowest complexity level whilst remaining physiologically relevant. The second step is the quality control of the data as described in Support Protocol 2 (Fig. 2). The third and final step is the characterization of the angiogenic sprouts using automated confocal imaging and high-resolution microscopy followed by quantitative automated methods using CellProfiler and ImageJ described in Parts 6 and 7. (Fig. 3). The analysis pipeline describes image segmentation of vessel sprouts including nuclei (Fig 3A, I-VI). The matrix-specific sprouting response (Fig. 3B) was characterized and quantified for fibrin and type I collagen (Fig. 3, C-G), see understanding results section. Altogether we provide a step-by-step strategy for inducing sprouts from hiPSC-ECs and their characterization in a microfluidic device.

Part 5: Angiogenic stimuli

Materials

- Type I collagen-filled or fibrin-filled, fibronectin-coated idenTx40 plate with a confluent hiPSC-EC monolayer (parts 1-4)
- EGM-2 (PromoCell, cat. C22211see recipe section)
- Angiokine-enriched EMG-2 (see recipe section)
- VEGF (165) IS, premium grade (Miltenyi Biotec, cat. 130-109-386)
- bFGF (Miltenyi Biotec, cat. 130-093-842)
- DAPT, γ-secretase inhibitor (5 mg, Sigma-Aldrich, cat. D5942)
- Sphingosine 1-phosphate (S1P) (Echelon Biosciences, cat. S-2000)

- Eppendorf® Multipette E3 pipette (Eppendorf, cat. 4987000010)

- Combitips Advanced® 2,5 ml (Eppendorf, cat. 0030089650) or Combitips Advanced® 5,0 ml (Eppendorf, cat. 0030089669)

Procedure

1. Prepare angiokine-enriched EMG-2 (see recipe section) 30 min before the stimulation of hiPSC-ECs.
2. Aspirate the medium from all reservoirs carefully.
3. Add 70 µL and 50 µL of EGM-2 into the upper left and right media ports, of the hiPSC-EC-seeded channel, respectively.
4. Apply the angiogenic stimuli by adding 70 µL of Angiokine-enriched EMG-2 into the lower left media ports and 50 µL of Angiokine-enriched EMG-2 into the lower right media.

Always try to avoid generating microbubbles inside the media channels. Guidelines for media refreshment, including pipette positioning, are described in the idenTx40 handling protocol v.1.2 (Aim Biotech, Singapore).

5. Repeat steps 1-5 every 24 hours for 2 days
6. Position the plate in a static flow condition to allow passive diffusion of the angiokine gradient at 37°C and 5% CO_2 (Fig. 1C).

Part 6: Characterization and quantification of the hiPSC-EC sprouts.

This protocol describes a semi-automated pipeline to assess 3D sprouting in AoCs in three steps using immunofluorescence staining. First, fixation and permeabilization of the hiPSC-ECs is followed by blocking of non-specific binding and the addition of primary antibodies. We used CD31 as an EC-specific adhesion marker, Phalloidin to visualize F-actin filaments (especially in tip cell filopodia), and SOX-17 as an EC-nuclear marker. The selection of secondary conjugated antibodies can be customized, and the dilutions used were according to the manufacturer's recommendation. Second, the stained 3D sprouts in the idenTx40 platform are well-suited for automated confocal imaging and high-resolution microscopy (Fig. 1D-C). Third, the quantification of the parameters from the

maximum projection images is performed using a CellProfiler and ImageJ as described below.

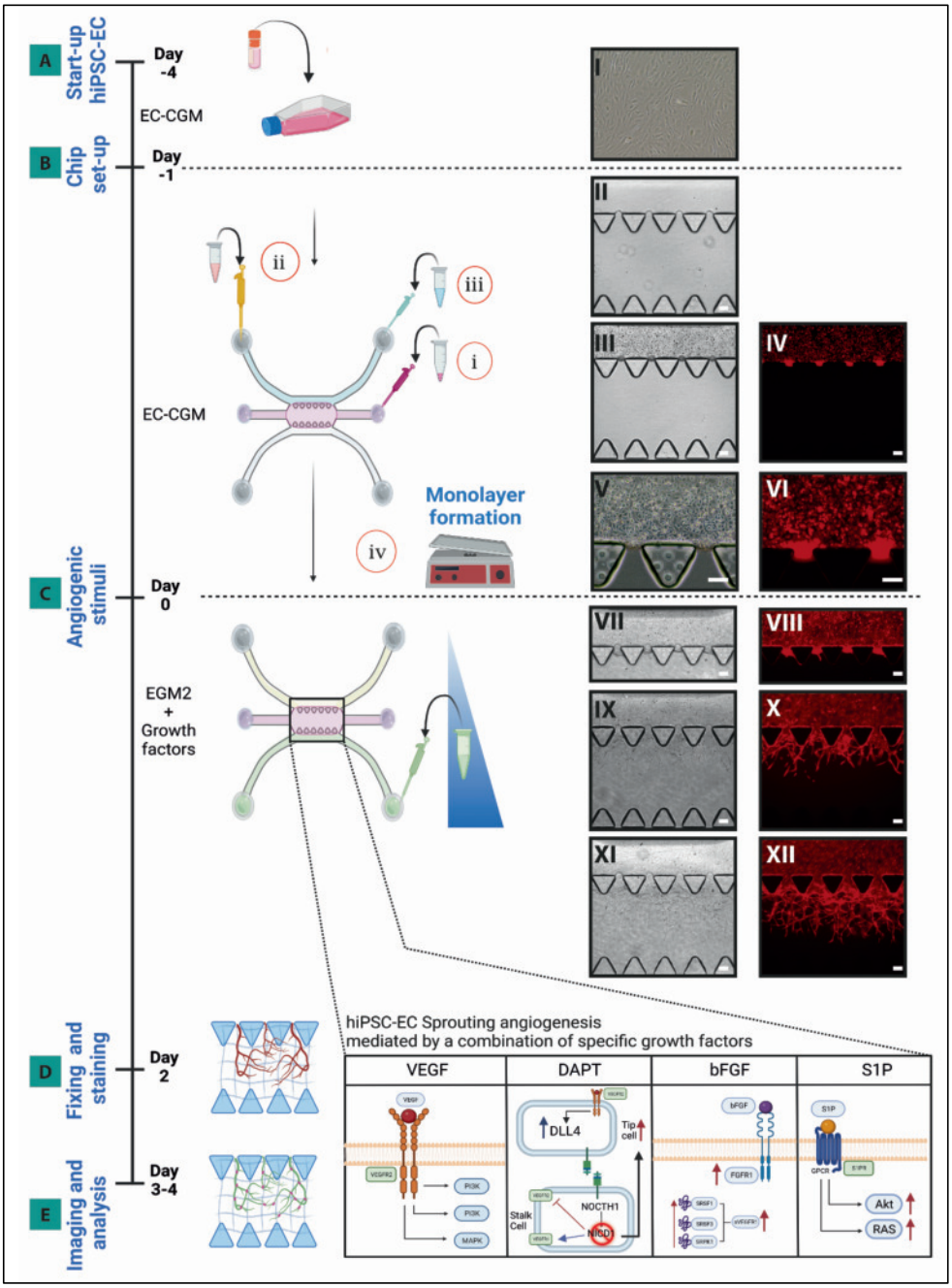

Figure 1. Schematic overview of the hiPSC-EC-based angiogenesis-on-chip assay and representative images of the step-by-step protocol. (A) Support Protocol 1 describes the expansion of hiPSC-ECs in a cell-line-

specific format 3 days prior to AoC set-up until ~80% confluency of the hiPSC-ECs is observed (I). (B) The Basic Protocol 1 corresponds to the Hydrogel preparation and filling of the middle gel channel (i, II; Parts 1 and 2 of the protocol) followed by Protein surface coating of the upper media channel with fibronectin (ii, II; part 3 of the protocol). Subsequent primary vessel formation in the top media channel using a standard hiPSC-EC density (iii; Part 4 of the protocol). Representative images post hiPSC-EC seeding (III-IV), and cell attachment in the top media channel after 2 hours (V-VI). The monolayer is enhanced over 24 hours by applying transversal gravity-driven flow using a rocker set at 5° degrees angle change every 8 minutes (iv). Representative images of the formed monolayer before angiogenic stimulation (VII-VIII). (C) The Basic Protocol 2 gives details about the angiokine-mediated stimulation of the hiPSC-ECs (Part 5 of the protocol) to induce sprouting angiogenesis (IX-X, shows hiPSC-EC sprouts at day 1; and XI-XII, at day 2) using 100 ng/ml VEGF, 20 nM DAPT, 500 nM S1P, and 40 ng/ml bFGF. Intracellular activation of VEGFR2, DLL4, NOTCH1, FGFR1, and S1PR by its ligands display the angiogenic sprouting capacity of the hiPSC-ECs. (D-E) Characterization of the 3D sprouting by immunofluorescence staining, high-content confocal imaging and analysis (Parts 6 and 7 of the protocol). (I, II, III, V, VII, IX, XI) Bright-field images (IV, VI, VIII, X, XII), and fluorescent live-cell images of hiPSC-ECs expressing mCherry (Red). (I-IV and VII-VIII) 4X and (V-VI) 10X objective. Scale bars, 100 μm. EGM-2, endothelial cell growth medium 2; EC-CGM, endothelial cell complete growth medium; VEGF, vascular endothelial growth factor; VEGFR2, vascular endothelial growth factor receptor 2; DAPT, γ-secretase and NOTCH inhibitor; DLL4, delta-like Notch ligand 4; NOTCH, Neurogenic locus notch homolog protein 1; bFGF, basic fibroblast growth factor; FGFR1, Fibroblast Growth Factor Receptor 1; S1P, sphingosine 1-phosphate, and S1PR sphingosine-1-phosphate G protein-coupled receptor. Created with BioRender.com

Part 6.1: Fixation and Immunofluorescence staining

Materials

- IdenTx40 Plate with 3D sprouts derived from hiPSC-ECs (Basic Protocol 2)
- 4% (w/v) Paraformaldehyde (PFA) see recipe section.
- 0.5% (v/v) Triton X-100 in DPBS (see recipe section)
- Blocking solution, 2% (w/v) BSA in DPBS (see recipe section)
- Storage solution, 1% (v/v) Pen/Strep in DPBS (see recipe section)
- DPBS (1X, Life Technologies, cat. 14190)
- Primary antibodies:

  Anti-human CD31, Mouse, 1:500 (DAKO, cat. M0823)

  Anti-human SOX17, Goat, 1:750 (R&D systems, cat. AF1924)
- Phalloidin-488, 1:1000 (Invitrogen, cat. A12379)
- Secondary antibodies:

  Donkey anti-goat Alexa Fluor 647, 1:300 (Invitrogen, cat. A21447)

  Donkey anti-mouse Alexa Fluor 594,1:300 (Invitrogen, cat. A21203)
- DAPI, 1:1000 (Invitrogen, cat. D3571)

- Parafilm sealing film, 10 cm × 38 m (Parafilm M, cat. 291-1213)
- Eppendorf® Multipette E3 pipette (Eppendorf, cat. 4987000010)

- Combitips Advanced® 2,5 ml (Eppendorf, cat. 0030089650)
- Combitips Advanced® 5,0 ml (Eppendorf, cat. 0030089669)

Procedure

7. Aspirate the angiokine-enriched medium from all the idenTx40 media reservoirs.
8. Add 50 µL of 4% PFA to all media reservoirs and incubate the plate for 30 min at room temperature.
9. Aspirate the PFA from the media reservoirs.
10. Wash the media channels by adding 100 µL of DPBS to all medium ports three times, 10-15 min each wash on the rocker.

To store the plate for longer than one month, wash it a fourth time with storage solution (see recipe section) and seal the plate with parafilm. The plate can be stored for three to six months at 4°C.
CAUTION: Work in a chemical hood.

11. Add 50 µL of 0,5% Triton X-100 to all media ports and incubate for 15 min at room temperature on the rocker to permeabilize the cell membrane.
12. Aspirate the Triton X-100 from all media reservoirs.
13. Add 50 µL of blocking solution (2% BSA in DPBS) to all media ports for non-specific binding site blocking.
14. Incubate for 2 hours at room temperature on the rocker.
15. Add 35 µL and 50 µL in the left and right media ports, respectively, the primary antibody mix (see recipe section).
16. Incubate primary antibodies overnight at 4°C.
17. Aspirate the primary antibodies from the media reservoirs
18. Wash three times by adding 100 µL of DPBS to all media ports for 5 minutes on the rocker.
19. Add 35 µL and 50 µL of the secondary antibody mix (see recipe section)to the left and right media ports, respectively.
20. Incubate for 2 hours at room temperature on the rocker.

It is recommended to use only Phallodin-488 and DAPI for non-phenotypic screening to optimize antibody usage. This process is faster, cost-effective, and gives a valid output for sprouting quantification (see recipe section).

21. Aspirate the secondary antibodies from the media reservoirs
22. Wash two times by adding 100 µL of DPBS to all media ports for 5 minutes on the rocker.
23. Aspirate the DPBS from the media reservoirs, add storage solution (100 µL in all media ports), seal the plate with parafilm, cover it with aluminum foil, and store it at 4°C.

The stained plate can be imaged within the next 30 days. Avoid dehydration and contamination of the channels by following step 23.

Part 6.2: high-content imaging

An in-house customized plate layout was set up in the high-content confocal system ImageXpress® Micro Confocal High-Content Imaging (Molecular Devices) for the idenTx 40 plates. The 40 microfluidic channels are imaged using the following parameters a 10X dry magnification objective and a 60µm pinhole. The maximum projection images (MIP) are generated automatically from the series of stacks images (260 µm, 2 µm stack size) of a single region covering ~80% of the microfluidic channel. For higher-resolution imaging, we recommend using the DragonFly spinning disk (Andor500) with the 10, and 40 magnification objectives. Perform image processing using Imaris 9.5 software (Bitplane, Oxford Instruments).

We recommend using the high-content imaging platform for faster imaging. All 40 channels can be recorded over ~2 hours (three to four colors).

Part 7: Analysis

Here, we describe an automated analysis strategy to study the morphological parameters of vessel sprouting as previously described. (Cuenca et al., 2021; Orlova et al., 2022; Arslan

et al., 2023) to obtain vessel density, number of hiPSC-ECs, branching point density, vessel length, and mean vessel diameter from 3D sprouts in type I collagen and fibrin.

Procedure

24. Store raw MIP images produced by the ImageXpress® microconfocal microscope.
25. Sort and transform MIP images into 8-bit format with ImageJ ("input images")
26. Run input images through the pipelines created with the free and open-source software CellProfiler (https://cellprofiler.org/) (Carpenter et al., 2006)

For vessel morphology, both CD31 and F-Actin channels were used as input. Besides the cropping step as mentioned for DAPI or SOX17, the median filter was applied to remove noise followed by a Gaussian filter. Image intensity was measured, and subtraction of the lower quartile intensity was performed to remove undesired extra noise produced by illumination effects. Next, image segmentation of the sprouts was performed using the threshold option (global strategy and the minimum cross-entropy method) to generate binary images (binarized or threshold image) and the vessel density readout (Fig. 3A I-IV).

To quantify the number of positive SOX17 or DAPI hiPSC-EC nuclei, pre-processing strategies were applied to crop images (from ~250 to ~1500 image coordinates), enhance feature speckles, and Gaussian filter to smooth edges, and remove unspecific noise. Primary objects were detected, converted into images, and quantified per microfluidic channel (Fig. 3A, V-VI).

27. Run the DiameterJ plugin in ImageJ and select the binarized threshold images as "input" (https://imagej.nih.gov/ij/, https://imagej.net/DiameterJ) (Hotaling et al., 2015)

SUPPORT PROTOCOL 2: Quality control of the platform based on hiPSC-ECs

Before the analysis, the maximum projection images of the microfluidic channels were subjected to the following inclusion criteria (Fig. 2A):

1) The type I collagen matrix must occupy the gel-filled channel and remain attached to the side posts.
2) Sprouts should surpass the limit of the upper posts regardless of their length.
3) Homogeneous sprouts develop throughout the inter-post spaces.
4) The gel channels should not contain > 25 cells unattached from the main sprouts.

The channels that met the four criteria, pass the first quality control step and are valid for further analysis (Fig. 2B, sprouts in Fibrin; and 2C, sprouts in type I collagen).

We recommend including the quality control metrics as an intermediate step between image acquisition and analysis, which must be performed for every cell line, and/or in the event of any change during the induction of sprouting angiogenesis. The technical success and absolute success are shown in Table 2 for both type I collagen and fibrin matrix, and are calculated as follows:

$$\boldsymbol{Technical\ success} = \frac{\boldsymbol{viable\ channels}*}{\boldsymbol{Total\ idenTx\ 40\ channels\ (40)}}\ \boldsymbol{x\ 100}$$

*Viable channels are the channels that were correctly filled with the matrix, fibronectin-coated, seeded with hiPSC-ECs, and formed monolayer over 24 hours ready to be stimulated.

$$\boldsymbol{Absolute\ success} = \frac{\boldsymbol{Channels\ meeting\ inclusion\ criteria\ +\ controls}}{\boldsymbol{Total\ idenTx\ 40\ channels\ (40)}}\ \boldsymbol{x\ 100}$$

Regarding the quality assurance of the assay, the signal window (SW) and Z'-Factor should be calculated for every hiPSC line (Table 2). Also, the coefficient of variation (CV) expressing the spread of the data gives important information for the selection of control lines. These metrics are used as recommended by NIH in the manual for "*Advanced assay development guidelines for image-based high content screening and analysis*". First, the number of replicates can be up to 7, but it is cell behavior-dependent and empirical. The assessment of the number of replicates per cell line/condition/clone must be determined by a first screening in duplicates and depending on the biological response of the treatment. (Bray and Carpenter, 2017) We recommend using at least five to eight replicates per condition/cell line/clone in addition to the negative controls.

Next, we calculated one of the most widely used quality metrics, the Z'-Factor, which represents how well separated the negative or control sample are from the positive control or treated sample (Table 2). Below the standard formula to calculate the Z'-Factor is shown (Iversen et al., 2006; Bray and Carpenter, 2017) and interpret the obtained values (Table 3). The most important limitation of the Z'-factor is the dangerous effect of the variation of the data. Therefore, due to the complex readout of the AoC, we decided to implement the robust Z'-Factor formula that uses the median and the median absolute deviation (MAD) (Atmaramani et al., 2020) to circumvent the data spread bias.

$$\boldsymbol{Z'Factor = 1 - \frac{3(\sigma p + \sigma n)}{(\mu p - \mu n)}} \quad \rightarrow \quad \boldsymbol{roboust\ Z'Factor = 1 - \frac{3(MADp + MADn)}{(Mp - Mn)}}$$

Where μ and σ represent the mean and standard deviation, M and MAD represent the median and median absolute deviation, and *p* and *n* represent the treated sample and negative control, respectively.

In addition, two extra parameters of assay quality that evaluate sampling distribution are recommended to calculate, the signal window (SW) (Table 2) and coefficient of variation (CV) (Table 4). Of note, individual metrics do not characterize the overall assay performance. Thus, a combination of the widely accepted Z'-Factor, CV, and SW provides a better understanding of the readouts and ensures the reliability of the data for further analyses (Chen et al., 2016; Iversen et al., 2006). The formulae to calculate the CV and SW are:

$$\boldsymbol{SW = \frac{\mu p - \mu n - (\sigma p + \sigma n)}{\sigma p}} \qquad \boldsymbol{CV = \frac{\sigma p}{\mu p - \mu n}}$$

Where μ and σ represent the mean and standard deviation, and *p* and *n* represent the treated sample and negative control, respectively.

It is also important to extract the descriptive statistics, such as de median, mean, and standard deviation, which allows in-depth details of the functionality range in which the hiPSC-ECs display an angiogenic response to be obtained. This is needed for cross-comparisons among conditions, cell lines, hydrogels and treatments. This information can be obtained separately per parameter (Table 5). Notably, in some cases, the validation of

these parameters needs to be double-checked as outliers, imaging, or analysis artifacts might not describe the phenotype observed in the representative figures.

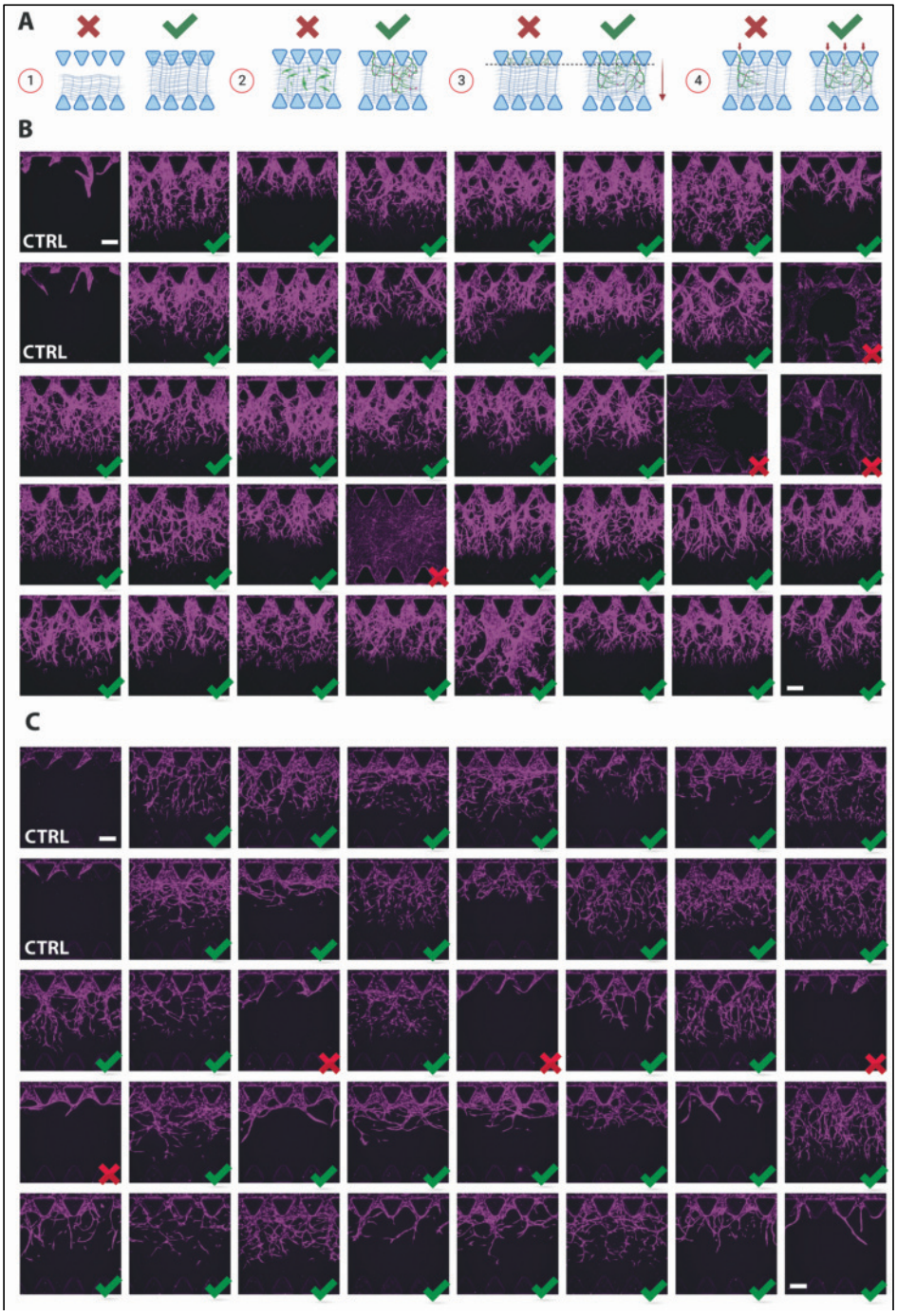

Figure 2. Inclusion Criteria and Quality Control of the AoC Assay. (A) Image pre-processing selection based on inclusion criteria as follows: (1) gel attachment; (2) minimum sprout length; (3) homogeneous sprouting; and (4) limited number of unattached endothelial cells (described in Support Protocol 2, inclusion criteria). Representative immunofluorescence images of the sprouting angiogenesis of CTRL#1 hiPSC-ECs in Fibrin (B) and type 1 collagen (C, alternative protocol) showing the full plate layouts containing 40 microfluidic channels. CTRL, channels in the control condition (EGM-2 added in the upper channel only without additional growth factors), and 38 angiokine-stimulated replicates. Green check (✓), channels that meet the inclusion criteria; Red mark (X), excluded channels. Representative images at day 2 post angiogenic stimuli. CD31, magenta (10x). Scale bars: 100 µm. EGM-2, endothelial cell growth medium 2.

Table 2. Quality control metrics of the angiogenesis-on-chip assay using CTRL #1 hiPSC-ECs in type I collagen and Fibrin.

	Z'-Factor		Signal window		Technical success	Absolute success
	Vessel density	Vessel length	Vessel density	Vessel length		
Type I collagen	0,11	0,31	1,56	3,01	91%	66%
Fibrin	0,27	0,18	1,4	2,94	94%	88,30%

Table 3. Interpretation of the Z'-Factor and Signal Window (SW) values.

	Value	Interpretation (Iversen et al., 2006)	Interpretation (Bray and Carpenter, 2017)
Z' -Factor	Z' = 1	-	An ideal assay
	1 > Z' ≥ 0.5	Excellent	Excellent: Good separation between the populations
	0.5 > Z' ≥ 0	Do-able	Marginal: Moderate to no separation of the distributions
	Z' = 0	Yes/No Assay	Nominal: Good only for a yes/no response
	Z' < 0	Unacceptable	Unacceptable: Screening is essentially impossible
SW	SW > 2	Recommended	
	SW >1	Acceptable	
	SW < 1	Unacceptable	

Table 4. Coefficient of variation of CTRL hiPSC-ECs across readouts.

	Type I collagen			Fibrin
	CTRL #1	CTRL #2	CTRL #3	CTRL #1
Vessel density	35,36%	28,16%	28,73%	30,34%
Mean vessel diameter	13,79%	14,50%	11,88%	15,98%
Branching point density	44,94%	43,24%	39,56%	41,64%
Normalized vessel length	43,46%	30,38%	28,02%	32,01%
Number of hiPSC-ECs	39,71%	32,81%	31,19%	46,39%

Table 5. Descriptive values of CTRL #1 performance in type I collagen and Fibrin.

		Mdn	mean	Sd
Vessel density (%)	Type I collagen	23,18	23,18	8,19
	Fibrin	30,68	30,36	9,21
Mean vessel diameter (µm)	Type I collagen	18,88	15,59	2.13
	Fibrin	15,88	16,46	2,63
Branching point density/µm^2)	Type I collagen	0,0002	0,0001	7,07E-05
	Fibrin	0,0011	0,0011	4,69E-04
Normalized vessel length	Type I collagen	8,00	7,20	3,47
	Fibrin	9,57	9,00	3,08
Number of hiPSC-ECs	Type I collagen	206	222	88
	Fibrin	287	285	132

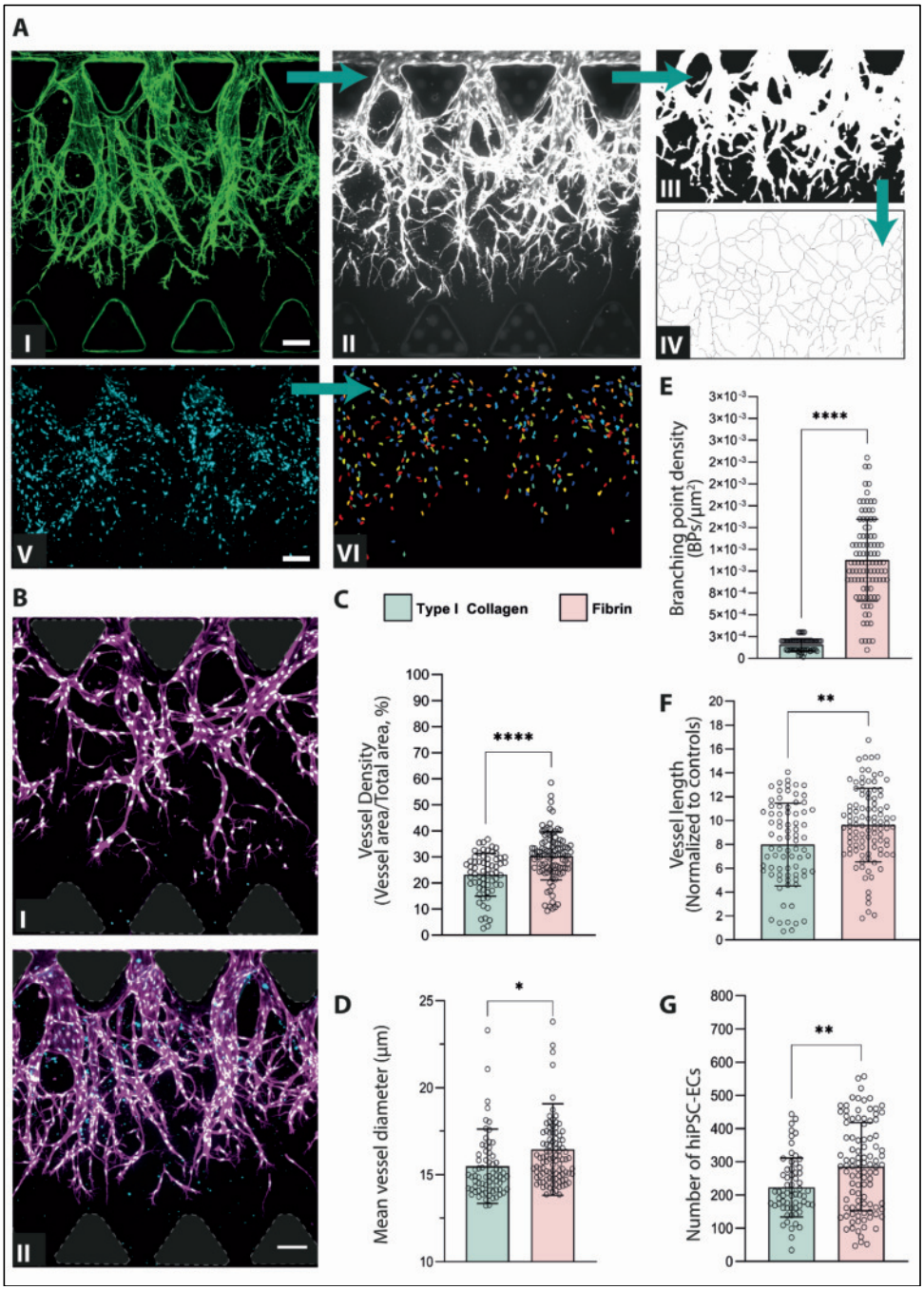

Figure 3. Characterization of the sprouting angiogenesis differences of CTRL #1 hiPSC-ECs in type I collagen Vs fibrin. (A) Immunofluorescence images of F-Actin (I, green) and SOX17 (V, cyan) at day 2 post angiogenic stimuli. Images were converted to 8-bit format and used as input for image binarization (II), skeletonization (III), and in the case of the nuclei, primary object counting (VI). Segmented images (I and V) show sprout morphology and the number of SOX17 positive nuclei (Basic Protocol 2, Parts 6 and 7). (B, I) Representative immunofluorescence images of sprouts in collagen matrix (1.5 mg/ml final concentration, w/v, see type I

collagen mastemix recipe section). (B, II) Representative immunofluorescence images of sprouts in fibrin matrix (2.5 mg/ml final concentration w/v, see fibrinogen recipe section). CD31, magenta; SOX17, white. Scale bars: 100 µm (10x). Quantification of (C) vessel density (%), (D) mean diameter (µm), (E) branching point (BP) density (BPs/mm2), (F) normalized vessel length to controls, and (G) number of hiPSC-ECs are shown (Part 7 of the protocol). Data are shown as ±SD from N = 3; three independent experiments with n = 18-31 and n = 26-39 microfluidic channels per experiment in type I collagen and fibrin, respectively. Unpaired t-test was used for comparison of data obtained from experiments in type 1 collagen. *p < 0.05, **p < 0.001, ***p < 0.0001. Scale bars: 100 µm (10x).

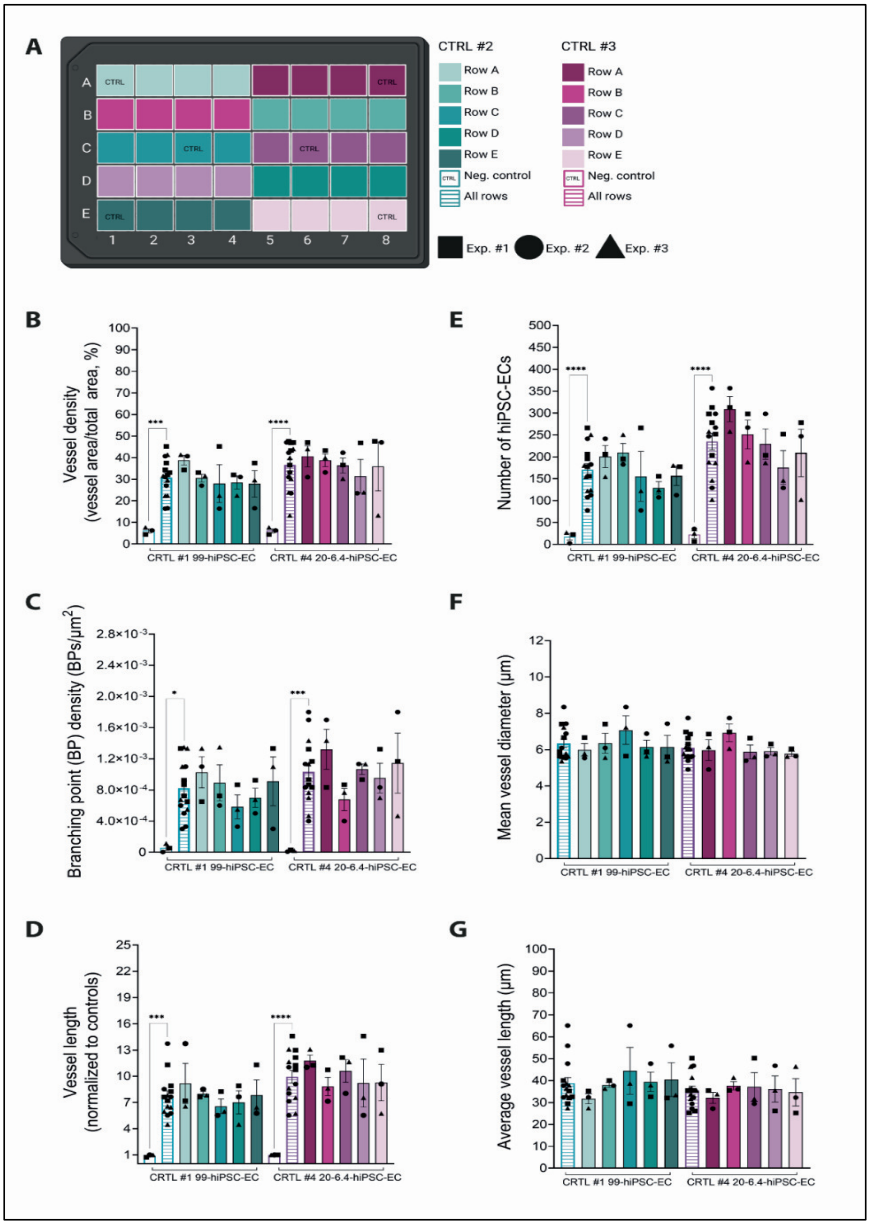

Figure 4. Assessment of idenTx40 plate position effect on sprouting angiogenesis. (A) Plate layout and CTRL #2 and #3 hiPSC-ECs specific position. "CTRL" indicates the control condition (EGM-2-only in the top media channel). Quantification of (B) vessel density (%), (C) branching point (BP) density (BPs/mm2), (D) normalized vessel length to controls, (E) number of hiPSC-ECs are shown, (F) mean diameter (μm), and (G) average vessel length (μm) are shown. Data are shown as ±SD from N = 3, n = 15; three independent experiments with 15 microfluidic channels per condition/experiment, each symbol representing five replicates. Two-way ANOVA (B-G) with Tukey's multiple comparisons. *p < 0.05, ***p < 0.0001

REAGENTS AND SOLUTIONS RECIPES

Gelatin solution, 0.1% (w/v)

As previously explained, 1 g of type A porcine skin gelatin (Sigma-Aldrch, cat. no. G1890) is dissolved in 100ml, autoclaved, diluted 10x in DPBS, and sterilized with a 0.22-μm membrane filter. The volumes needed for gelatin coating are the following:

Cultureware format	Gelatin 0.1%
6-well	1 mL
T75 flask	5 mL

Endothelial Cell Complete Growth Medium (EC-CGM)

EC-CGM recipe has been previously established and tested for hiPSC-EC expansion (Orlova 2014b. 2014a). To prepare 250 ml of EC-CGM, mix the following reagents: EC-SFM (Gibco 11111-044), Human serum from platelet-poor plasma (hPPS, Sigma-Aldrich/P2918), Human bFGF, premium grade (Miltenyi Biotec/130-093-842), Human VEGF 165 IS, premium grade (Miltenyi Biotec/130-109-386), as shown in the table below. Sterilize by filtering with a 0.22-μm membrane filter. Store the EC-CGM at 4 °C and use within 2 weeks.

Reagents	**Volume**	**Final concentration**
EC-SFM	247.5 mL	-
hPPS	2.5 mL	1%
VEGF, 50 μg/mL	150 μL	30 ng/mL
bFGF, 100 μg/mL	50 μL	20 ng/mL

Endothelial Cell Growth Medium 2 (EGM-2)

To prepare 500 ml of EMG-2 (PromoCell, cat. C-22211), add the supplements from the SupplementPack to the basal medium as specified by the manufacturer. In addition, add 2.5 ml Penicillin-Streptomycin (5000 U/ml; GibcoTM; Thermo Fisher Scientific, cat. no. 15070063). Keep the EGM-2 at 4 °C and use within 6 weeks. EMG-2 contains the following compounds that might influence angiogenesis:

Compound	Final concentration in EMG-2
Fetal Calf Serum	0.02 ml/ml
Epidermal Growth Factor (recombinant human)	5 ng/ml
Basic Fibroblast Growth Factor (recombinant human)	10 ng/ml
Insulin-like Growth Factor (Long R3 IGF, recombinant human)	20 ng/ml
Vascular Endothelial Growth Factor 165 (recombinant human)	0.5 ng/ml
Ascorbic Acid	1 µg/ml
Heparin	22.5 µg/ml
Hydrocortisone	0.2 µg/ml
Penicillin-Streptomycin*	0.5 %

*Extra supplement not suggested by the manufacturer.

Type I collagen master mix, 1.5 mg/ml (w/v)

The type I collagen master mix is sufficient to fill 1 idenTx40 plate. The optimal final pH of the solution is ~ 7.4, identified with a light pink color. The type I collagen master mix should be kept on ice and used immediately upon mixing the following reagents: Medium 199 (10X, Gibco, Life Technologies, cat. 21180-021), $NaHCO_3$ (7.5 %, Gibco, cat. 25080-60), 1M NaOH solution (Merck, cat. no. Merck (cat. no. 1064981000).), HEPES (1M, Gibco, Cat. 15630-080), Sterile deionized water (Gibco, cat.15230-08) and type I Collagen, rat tail (5mg/ml, R&D Systems, cat. 3447-020-01).

Mixing order	Reagents	Volume (µL)
1	Medium 199 (10X)	66,66
2	dH_2O	359,26
3	NaHCO3 (7,5%)	19,54
4	HEPES (1M)	16,60
5	5 mg/ml Type I Collagen (w/v)	200
6	NaOH (1M)	4,60
	Total Volume	666,66

Fibronectin solution, 50 µg/ml (v/v)

Dilute the 50 µL of fibronectin bovine plasma (1 mg, Sigma-Aldrich, Cat. no. F1141-1MG, Lot SLBX2646) in 950 µL 1X DPBS (Life Technologies, Cat. no. 70011044), dilution factor 1:20. The solution must be sterile, no filtering is needed.

Fibrinogen solution, 5 mg/ml (w/v)

Dissolve 50 mg of Fibrinogen (Sigma-Aldrich, cat. F8630-5G) in 10 ml of DPBS (1X, Life Technologies, Cat. no. 70011044), at 37 °C in a water bath. Sterilize by filtering with a 0.22-µm membrane filter. Prepare 25 µl aliquots and keep them on ice.

Triton X-100, 0.5% (v/v)

Dilute 250 µl Triton X-100 (Sigma-Aldrich, cat. T8787) in 50 ml DPBS followed by placing the 50 ml falcon tube in a tube rocker for at least one hour. Sterilize by filtration using a 0.22 µm membrane filter. Store the solution at room temperature for up to 1 year.

Vascular endothelial growth factor (VEGF) stock solution, 50 µg/ml (w/v)

Reconstitute VEGF (165) IS, premium grade (Miltenyi Biotec, cat. 130-109-386) 0.1% (w/v) BSA in DPBS to a stock concentration of 50 µg ml-1. Store aliquots at −80°C and always handle them on ice. Avoid freeze-thaw cycles.

γ-secretase inhibitor DAPT, stock solution, 10 mM

Dissolved the DAPT (5 mg, Sigma-Aldrich, cat. D5942) in 1.1562 ml of Dimethyl sulfoxide (DMSO) to obtain a stock concentration of 10 mM. Store aliquots at −20°C and handle them on ice. Avoid freeze-thaw cycles.

Basic fibroblast growth factor (bFGF) stock solution, 20 *µg/ml (w/v)*

Prepare a first dilution of lyophilized bFGF (50 µg, Miltenyi Biotec, 130-093-842) to 100 µg/ml in distilled water (dH2O; Gibco™; Thermo Fisher Scientific, cat. no. 15230089). To reach a concentration of 20 µg/ml, dilute the first dilution using 0.1% (w/v) BSA in DPBS. Aliquot small volumes to avoid freeze-thaw cycles and store at -80 °C.

Sphingosine 1-phosphate (S1P) stock solution, 1 mM

Reconstitute 1 mg of S1P (Echelon Bioscience, S-2000) in 95% methanol and 5% warm acetic acid, vortex for 1 min and sonicate for 10 min until the solution is clear. The stock concentration obtained is at a concentration of 1 m. Store it at −20 °C use in a dilution factor 1:2000 (500 nM).

Angiokine-enriched EMG-2 (v/v)

Angiokines are added to the previously prepared EGM-2 medium (see recipe sections). This solution must be prepared under sterile conditions for cell culture, stored at 4°C, and used within 24 hours.

Reagents/stock [ ]	Volume	Relative concentration	Final concentration
EGM2	10 ml	-	-
VEGF 50 µg/ml	20 µl	100 ng/ml	105 ng/ml
bFGF 20 µg/ml	20 µl	40 ng/ml	50 ng/ml
DAPT 10 nM	20 µl	20 nM	20 nM
S1P 1 mM	5 µl	500 nM	500 nM

Blocking solution, 2% (w/v) BSA in DPBS

Dissolve 1 g bovine serum albumin (BSA) (Sigma-Aldrich, cat. A3311) in 50 ml DPBS (1X, Life Technologies, Cat. no. 70011044) by tube rocking for 1 hour. Sterilize by filtration using a 0.22 µm membrane filter. Store the solution at 4°C and use it within 6 months.

Primary antibody mix

Dilute the blocking solution to obtain 1% (w/v) BSA in DPBS. Prepare 7.5 ml of the mix to obtain enough volume to fill one idenTx 40 plate. Dilute the antibodies in 1% (w/v) BSA in DPBS using the following dilutions.

Reagents	Dilution factor	Volume
Anti-human CD31 (DAKO, cat. M0823)	1:500	15 µL
Anti-human SOX17 (R&D systems, cat. AF1924)	1:750	10 µL
Phalloidin-488 (Invitrogen, cat. A12379)	1:1000	7.5 µL
1% (w/v) BSA in DPBS	-	7.47 ml

Secondary antibody mix

Prepare 7.5 ml of the mix to obtain enough volume to fill one idenTx 40 plate. Dilute the secondary antibodies in 1% (w/v) BSA in DPBS using the following dilutions.

Reagents	Diltuion factor	Volume
Alexa Fluor 594, Donkey anti-mouse (Invitrogen, cat. A21447)	1:300	25 µL
Alexa Fluor 647, Donkey anti-goat (Invitrogen, cat. A21203)	1:300	25 µL
DAPI (Invitrogen, cat. D3571)	1:750	10 µL
1% (w/v) BSA in DPBS	-	7.40 ml

Storage solution, 1 % Pen/Strep (v/v) in DPBS

Add 5 ml of Pen/Strep, Penicillin-Streptomycin (5000 U/ml; GibcoTM, cat. 15070063) to 500 ml of 1X DPBS (Life Technologies, Cat. no. 70011044). Mix and store at 4 °C. Use this solution within the next six months.

COMMENTARY

Background information

Angiogenesis is a complex series of events that results in the expansion of the vascular network. Recapitulating angiogenic processes in a single microphysiological system is currently limited by the low reproducibility and scalability lack of flow-induced stimulus, and the nature of the ECM as well as the type of ECs used. Conventional 2D in vitro vascular assays like the scratch assay and tube formation are simple to evaluate some of the key angiogenic processes, but they do not recapitulate how sprouting occurs in 3D in tissues (Staton et al., 2009; Stryker et al., 2019). Some 3D assays like vascular spheroids do allow in-depth study of the tip/stalk cell phenotype, lumen, and cell retrieval for further functional studies, but lack perfusion and the ability to create an angiokine gradient and they have random spatial cell growth and distribution, and high reagent usage (Davis et al., 2013; Pfisterer and Korff, 2016; Heiss et al., 2015; Kim et al., 2023). Altogether, these assays lack the complexity needed to recapture angiogenesis fully, which creates the need for platforms that do incorporate these features.

Microfluidic systems are emerging as technologies that faithfully mimic tissue architecture. Robust modeling of 3D sprouting angiogenesis using primary ECs has been previously described, and this allows selective spatial control of cells and angiokine gradient. The sprouting response of HUVECs to VEGF-165, S1P, and phorbol 12-myristate 13-acetate (PMA) in type I collagen-filled hydrogel in the middle channel of a high-throughput microfluidic device has for example been described and concluded that this growth factor combination was optimal for this cell type (Duinen et al., 2019). Another study described a low-throughout PDMS-based device, in which the HUVECs first formed a 3D network in 10 mg/ml of fibrin with a cell suspension of 6.7x10^6 cells/ml combined with fibroblasts, followed by sprouting induction in a parallel channel filled with 2.5 mg/ml of fibrin using only VEGF and S1P (Kim et al., 2016). The phenotypic shift from quiescent vasculature towards an angiogenic phenotype served as an accurate active vasculature model although its complexity hinders the scalability. Of note, a model closer to that described here is where the fibrin matrix is placed in the middle gel channel together with supporting cells and HUVECs in the side channel, which showed that

doxycycline-induced SOX17 ECs displayed a hypersprouting phenotype with pericytes (Kim et al., 2023). However, this method is low throughput, limiting compound testing and the use of patient-derived ECs for personalized disease modeling.

The emergence of hiPSC is of particular interest for human disease models, as they are a renewable source of cells and can be differentiated into most cell types of the body, including ECs. We previously described a protocol to efficiently induce EC differentiation from hiPSCs through mesoderm specification under defined culture conditions and carried out extensive functional characterization (Orlova et al., 2014). We further demonstrated the potential utility of these hiPSC-ECs to develop a macrovascular 3D model combined with mural cells coupled with flow to study vascular barrier function (Bulut et al., 2022). In addition, we employed hiPSC-ECs from patients with Hereditary Hemorrhagic Telangiectasia (HHT), a genetic vascular disease, to model vasculogenesis in health and disease (Orlova et al., 2022). The transition from primary ECs to hiPSC-ECs demonstrated feasibility in human models of angiogenesis that use high-throughput platforms (Duinen et al., 2019). In addition, hiPSC- ECs selectively sense angiogenic inhibitors such as Sunitinib and 3PO, showing the advantage and higher sensitivity of hiPSC-derived ECs for drug testing (Duinen et al., 2020) Other studies using HUVECs for angiogenesis were converted to high-throughput screens for antiangiogenic compounds (Soragni et al., 2023; Kim et al., 2021)

Currently, AoC applications have been shown most suite to testing proangiogenic or antiangiogenic angiokines or compounds (Stryker et al., 2019). Of note, recent evidence suggests that high-throughput phenotypic testing of antiangiogenic kinase inhibitors using microdevices coupled with automated imaging and analyses can accelerate the drug testing pipeline (Soragni et al., 2023). Therefore, this protocol focuses on the incorporation of hiPSC-ECs into one of two matrices, fibrin and type I collagen. Both, Fibrin and some types of collagens contain RGD sequences that bind to integrins α5β1, αvβ3, and α2β1, which differentially modulates hiPSC and induce different angiogenic responses (Feng et al, 2013; Nicolas et al., 2020). Also, fibrin is pro-inflammatory, sequesters growth factors, and increases vascular permeability and leakage (Côté et all, 2016; Dvorak, 2015). The hiPSC-EC interaction with the ECM influences the reproducibility and the phenotype of the sprout. Its selection depends on the research

question, cell type and assay performance. We aimed to elucidate how hiPSC-ECs respond to different matrices. Additionally, we have included guidelines for quality control towards standardizing the hiPSC-EC-based AoC assay.

Critical parameters

It is important to properly start up the hiPSC-ECs, and to expand them until 80% confluency is reached (Support Protocol 1, Figure 1A). Otherwise, their functional sprouting capacity might differ among experiments and yield higher variation of the readouts. The hiPSC-EC suspension preparation step (Support Protocol 1) is also key, as variation in the cell density leads to different sprouting responses. We have observed that a cell density <4x10^6 hiPSC-ECs/ml results in cell detachment, impaired monolayer in the top medium channel, with thinner and fewer sprouts. On the other hand, a cell density higher than >5x10^6 hiPSC-ECs/ml results in unattached and dead cells, spontaneous sprouts, 2D cell growth underneath the gel, gel detachment, and in some cases, quiescent vessels unreactive to the angiokine-enriched medium (data not shown). Hence, we recommend working within this range when using hiPSC-ECs (Fig. 1, III-IV).

The chip setup represents the crucial part of the assay. The hydrogel selection should be based on the needs and research questions. We propose two hydrogels that have been tested in this platform, each requiring specific considerations. Type I collagen matrix is a pH- and temperature-dependent reaction, we recommend always working on ice and following the recipe section precisely. Since the final type I collagen concentration is low (1.5 mg/ml), we advise using a low-concentration stock of type I collagen (5 mg/ml). HEPES and $NaHCO_3$ act as buffers, and NaOH adjusts the pH to ~7.4, as described previously. If the final mastermix does not reach this pH, denoted by a pink color similar to the EMG-2 medium, it should not be used (see Troubleshooting table). HiPSC-ECs are sensitive to pH changes which affect cell survival and lead to reduced or quiescence vessels. Regarding the fibrin matrix, it is a more standardized matrix in which the ratio of fibrinogen to thrombin is critical. We recommend using a final concentration of 2.5 mg/ml of fibrinogen and 1 U/ml of thrombin. To reduce channel-to-channel variation, it is necessary to use 1 aliquot of mix fibrinogen-thrombin to fill a maximum of 5 channels. Also, all reagents should be kept on ice and not exceed the maximum times recommended.

Also, the coating step using a high concentration of fibronectin ensures not only proper cell attachment but also channel permeability. Occasionally, leakage of the matrices to the side media channels is observed, and when filling with the fibronectin coating solution, the channels can become unblocked and permeabilized. However, in some cases, this is not possible as matrix polymerization takes place, and the channels are occluded. It is crucial to properly flush the coating solution with medium, we have observed better cell adhesion and cell survival when the time of flushing of the coating solution using EC medium is extended for more than one hour. Regarding monolayer formation, it is optional to subject the plate to transversal flow (Part 4, step 33). However, we highly recommend implementing a 5° angle with an interval change of 8 min when using hiPSC-ECs. It is important not to exceed a 7° angle to avoid gel detachment, 2D proliferation, and single-cell migration. As a limitation, there is a percentage of channels expected to fail per experiment (Table 2), mainly because of the abovementioned causes, but in most cases, the technical success at this point should reach 97.5-100 %.

It is imperative to avoid air bubbles inside the microfluidic media channels during the EC stimulation (Fig. 1C, IX-XII). If air bubbles are in the bottom media channel, the transversal flow of the angiokine-enriched EGM-2 will be impaired, and the hiPSC-ECs will not properly sense the angiogenic stimuli. This will result in non-homogeneous, reduced, or inhibited sprouting. It is therefore important to check all the channels using an optic microscope every day and to follow the guidelines for troubleshooting (Table 6) in case air bubbles are observed. Lastly, the use of fresh angiokine-enriched EMG-2 is not critical; it can be prepared once for the two-day stimulation, but we recommend using recently prepared angiokine-enriched EMG-2. In some cases, and despite the chip set-up being optimal, the hiPSC-ECs are quiescence or fail to sense the angiogenic stimuli. If the amount of non-sprouting channels does not exceed 10% (4 channels), continue the assay and endpoint troubleshoot. Altogether, the assay reproducibility should be consistent although the angiogenic response of the cells is intrinsic.

Troubleshooting

Guidelines for troubleshooting are described in Table 6.

Understanding Results

Sprouting angiogenesis comprises multiple processes that are all mimicked in one assay. However, the deconvolution of this information is challenging, and the outcome depends on following standard procedures as mentioned above. The initial seeding density, which is ~5 x10^4 per channel, is the most crucial step to obtain consistent results independently of the matrix used. After 24 hours post-seeding, hiPSC-ECs should form an intact monolayer that covers the surface of the medium channel completely. At this point, initial tip cell specification is observed in the form of small protrusions into the matrix and the morphology of the ECs of the media channel (the primary vessel) should not appear altered. During the following 2-day sprouting induction with angiokines, the hiPSC-ECs rapidly activate, invading the gel towards the gradient, and in some cases, lumen formation is observed. However, the results obtained are dependent on several factors as described below.

First, the selection of fibrin versus type I collagen matrix depends on several considerations, but most importantly, it is important to take into account the pro-inflammatory effects of the fibrin on ECs, especially for disease modeling. Technically, gel filling with fibrin solution takes longer and requires more technical expertise to avoid cross-channel differences during the pipetting. However, the number of channels meeting the inclusion criteria is between 91% and 94% (Technical success), and 66% and 88% (Absolute success) in type I collagen and fibrin matrix, respectively (Table 2; Fig. 2B and 2C). The quality control metrics showed that the separation of the negative control and samples does not differ significantly between the hydrogels using vessel density and normalized vessel length parameters (Table 2, Z'-Factor and SW). Despite statistics showing no significant differences, we do observe that sprout development is faster on day 1 in fibrin than in type I collagen, showing more directionality towards the angiokine gradient. Both quality metrics report the readouts of this assay as "yes/no" or "do-able" (Table 2), meaning that the assay can answer yes/no research questions due to the moderate separation of the samples (Table 3)

It is worth mentioning the sample variation displayed by the hiPSC-ECs. Three control hiPSC-ECs (CTRL #1, 2, and 3) were tested in type I collagen and CTRL #1 in the fibrin

matrix; this showed lower coefficient of variation in the sprouting response of CTRL #1 in the fibrin matrix (Table 4). Despite the higher consistency of the angiogenic response in fibrin over type I collagen seen in the representative images (Fig. 2B and 2C), it is difficult to conclude that the fibrin matrix drives superior sprouting response since only one control hiPSC-EC line was tested in both hydrogels. However, the fibrin matrix was observed more robust and more promising for scalability using CTRL #1 hiPSC-EC. In-depth assessment of the 3D sprouts in different hydrogels is shown in Fig. 3B, where all the parameters (vessel density, diameter, branching point density, normalized vessel length, and number of hiPSC-ECs) are significantly higher in fibrin than in type I collagen (Fig. 4C-G). Since both hydrogels have intrinsic biophysico-chemical properties, it is important to know the angiogenic phenotype of each hiPSC-EC line and their sprouting behavior in the hydrogels. We observed that the most precise readout parameters are the vessel density, normalized vessel length, and the number of hiPSC-ECs (Table 5). However, other analysis pipelines can be adapted to extract other parameters from the MIP images but need further validation.

The reliability of the results depends on the minimum number of replicates used, which is usually a high number used to validate the observations (Fig. 3). We recommend using 5-8 replicates per line, clone, or condition to test. Further experiments were conducted to 1) evaluate if a lower number of replicates per condition impacted the readout and 2) determine any plate positioning effect (Fig. 4) using two different control hiPSC-EC lines. The gel filling, fibronectin coating, preparation of CTRL #2 and #3 hiPSC-EC suspensions, seeding, and stimulation were conducted independently. Also, two batches of differentiation were used to run three experiments. The results show that the position effect of the replicate in the idenTx40 platform is too low to observe significant differences among the locations and that using minimally 5 replicates after the quality control and inclusion criteria is sufficient for significant outcomes of analysis. We recommend determining the minimum number of replicates needed per experiment beforehand. Some parameters such as the number of nuclei and branching point density are not sensitive to the analysis while other parameters as vessel density and vessel length are more accurate. The use of hiPSC-ECs to model angiogenesis using microfluidic devices is feasible but carries a certain degree of complexity as outlined in these guidelines.

Time considerations

Basic Protocol 1: the chip set-up takes a total of 24 hours excluding the Support Protocol 1, which starts before the Basic Protocol 1.

Step 1: 3 hours until fibrinogen is dissolved in DPBS.

Steps 2-8: ~30 for filling of the idenTx40 plate with fibrin solution, including the 15 min polymerization time at room temperature.

Step 9 (Alternative): ~5 min to prepare the type I collagen matrix solution

Steps 10-17 (Alternative): ~20 min for filling of the idenTx40 plate with type I collagen solution, including 15 min polymerization time in the incubator.

Steps 18-21: ~10 min to coat the top media channel with fibronectin.

Step 22: 1 hour incubation time with fibronectin.

Step 23-25: 1 hour to allow proper flush of the fibronectin solution and plate stabilization.

→ *Pause point: the plate can be stored at 37°C for 24 hours prior to cell seeding.*

Steps 26-29: ~25 min to seed the top media channel with hiPSC-ECs excluding the cell suspension described in Support Protocol 1.

Steps 30: 2 hours to allow hiPSC-EC attachment.

Steps 31-33: ~5 min to inspect the cell attachment, addition of medium, and application of transversal flow using a rocker.

→ *Pause point: 24 hours to develop a confluent monolayer.*

Basic Protocol 2: Induction of sprouting angiogenesis, characterization, and analysis together takes approximately 4 days, detailed timing is described as follows:

Steps 1-6: ~30 min to prepare the angiokine-enriched sprouting media and plate refreshment.

Steps 7-10: 1 hour to fix and prepare the plate for staining.

→ *Pause point: The plate can be stored for up to a month before step 11.*

Steps 11-23: 24 hours to perform immunofluorescence staining, including 2 hours waiting time for serum blocking in step 14, overnight primary antibody incubation, and 2 hours for secondary antibody incubation.

Part 6.2: ~2 hours to image the 40 replicates of 1 idenTx40 plate.

Part 7, steps 24-27: ~5 hours to run the analysis and prepare a summary.

Support Protocol 1: the initial steps 1-9, for hiPSC-ECs expansion, take ~90min.

→ *Pause point: 3-5 days are needed to expand the endothelial cells needed.*

Steps 10-19***:*** ~30 min to preparation of hiPSC-EC suspension.

Support Protocol 2: The quality control of the hiPSC-ECs-based platform step is performed together with Part 7 (Steps 24-27).

Overall, from the initial EC expansion until sprout characterization and extraction of data, the protocol takes around 7 days.

Acknowledgments

This project was funded by the Netherlands Organisation for Health Research and Development (ZonMw) PTO446002501 grant and supported by the Novo Nordisk Foundation Center for Stem Cell Medicine (reNEW) from the Novo Nordisk Foundation grants NNF21CC0073729. Also, the Leiden University Medical Center (LUMC) hiPSC Hotel, the Light and Electron Microscopy Facility, and MSc. Rudmer Postma from Einthoven Lab in the Department of Nephrology of the LUMC for the high-content confocal imaging support.

Data Availability Statement

The corresponding author can provide access to the data, tools, materials, or protocol support upon request.

Table 6. Troubleshooting guidelines.

Part/step(s)	Problem	Possible cause (s)	Solution
Basic Protocol 1, Part 1	Clogged fibrin in tips and gel channels	Incorrect fibrin concentration	Discard and prepare new.
		Higher thrombin concentration	Use aliquots to fill only 3-5 channels and discard leftover
	Detachment of fibrin	Dryness of the channel	Consider adding EC-CGM in the top media channel and DPBS in the bottom media channel.
Part 2 (Optional protocol)	Yellow or magenta type I collagen mastermix	Incorrect pH	Discard mastermix and prepare a new one. Persistent problem #1: discard stocks of M199, NaOH, NaHCO3 and rat tail collagen Persistent problem #2: check the calibration of pipettes
	Leaking of gel solution to media channels	Uneven surface	Place the plate in a horizontal hood surface
		Combitip® positioning error	Position the Combitip® at 90° perpendicular to the plate occluding all the gel ports by exerting soft pressure
		Presence of microbubbles	Avoid microbubbles during mixing by pipetting up and down slowly
Part 4	Impaired hiPSC-ECs survival	Error in Support Protocol 2: High hiPSC-EC density	Check EC suspension, optimal range from 4-5x10^6 cells/ml
		Prolongated time between trypsinization and seeding	Use EC suspension within 30 minutes after preparation at room temperature or within 1 hour on ice

	Monolayer not formed	Suboptimal number of initial seeded cells	Adjust cell seeding per cell line/clone Increase the rocking time of the plate from 24 to 48 hours
		Cell death	Ensure proper washout of the fibronectin solution
Basic protocol 2, Part 5	No sprouts observed on day 1 post-angiogenic stimuli	Error is angiokine-enriched EGM2	Discard and prepare new EGM2 with fresh growth factors
		Presence of air bubbles in the bottom media channel	Aspirate carefully the media and bubbles present in the bottom channel, inject manually with a p20 15 µL of angiokine-enriched EGM2, and proceed to repeat step 5 of the basic protocol 2
	2D invasion underneath or above the hydrogel	Error in hydrogel preparation	Channel is not optimal for analysis and must be excluded. If more than 20 channels present 2D cell invasion, discard the plate.
	Hydrogel detachment	Increased volume of medium	Do not exceed 100 µl in the media reservoirs
		Excessive exposure to transversal flow (> 48hours)	Do not leave the plate > 48 hours in the rocker Do not use angles higher than 7° or shorter intervals than 8 min
Part 6.1	Failed staining	Low dilution factor of primary antibodies	Increase CD31 dilution factor to: CD31: 1:300; SOX17: 1:500; Phalloidin to 1:500
Part 7	Pipeline bugs	Error in several lines of the DiameterJ due to ImageJ.	Use ImageJ 1.5w, Java 1.8.0_172 (64 bits). Persistent problem: contact authors for guidance
Support protocol 1	Insufficient number of cells	Suboptimal starting cultureware format	Decrease well surface or increase initial starting cell density until reaching ~80% confluency at days 3-5
Support Protocol 2	Z'-Factor < 0	Cell line or condition not optimal for assessment	Assay not valid

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